EXPRESSION OF A HUMAN INSULIN PRECURSOR IN *P.PASTORIS*

BACKGROUND OF THE INVENTION

1. Field of the Invention.

The present invention relates to the expression of human insulin in *P. pastoris* and, more particularly the invention is related to the field of DNA recombinant technology and to the production of insulin precursors in host microorganisms such as yeast. More precisely, the invention refers to a recombinant methylotrophic yeast strain for producing human insulin precursors. The invention also relates to DNA constructions and ~~method~~methods for obtaining the strains. The inventive strain comprises, in its genome, at least one copy of a first DNA construction and one copy of a second DNA construction, wherein said constructions are capable of ~~conducing the expression~~expressing and ~~secretion of~~secreting an insulin precursor.

2. Description of the Prior Art.

It is well known that ~~the disease of Diabetes~~diabetes is usually treated with insulin injections ~~of insulin~~, e.g. of human insulin. Insulin is ~~a central~~an essential hormone ~~of the~~ in metabolism and is a protein consisting of two polypeptide chains, namely A chain and B chain. A chain comprises 21 amino acid~~s~~ residues and B

chain comprises 30 amino acid residues, and both chains are covalently connected by di~~-sulphur~~sulfide bridges in the positions A7-B7 and A20-B19, and by an intra-~~catenary di-sulphur bridge~~ chain disulfide bond connecting ~~the~~ residues A6-A11.

~~The insulin~~Insulin is produced in the pancreas by the ß cells of the Langerhans islets as preproinsulin. ~~The preproinsulin~~Preproinsulin consists of a prepropeptide having 24 amino acids ~~actuating~~acting as an export~~ing~~ signal sequence followed by a peptide named proinsulin and containing 86 amino acid residues. Said preproinsulin may be represented by: ~~prepetide~~prepeptide-B-C-A, wherein the C peptide is a ~~connector~~connecting peptide comprising 31 amino acid residues and chains A and B are ~~the~~ chains A and B of ~~the human~~ proinsulin.

When the preproinsulin chain is synthesized, the signal peptide directs ~~the~~ synthesis ~~into~~towards the endoplasmic reticulum of the ß cells~~, in~~ and at that moment the signal peptide ~~is split~~splits out, secreting the proinsulin into the endoplasmic reticulum.

Then, during ~~the~~ packing of the insulin molecule within the secreting system of the ß cells, the ~~peptide C is split secreting~~peptide is cleaved, thus liberating the native insulin molecule, ~~properly~~ which is appropriately "folded". ~~The split of~~ Cleavage of the C peptide ~~C~~ is

carried out ~~under~~ through the action of enzymes ~~actuating~~ acting upon the proinsulin dibasic sequences.

Presently it is known that the C peptide ~~C carries out~~ has an important function in the formation of the tertiary structure of the insulin molecule.

The production of insulin for treating ~~Diabetes is~~ diabetes has been a concern ~~for~~ in the pharmaceutical industry ~~since~~ for many years ~~ago. As from~~. Since the development of ~~the~~ recombinant DNA techniques, a wide variety of ~~method~~methods for the production of insulin in microorganisms ~~has~~have been published ~~in several media:~~.

~~The~~ Bacteria were the first host microorganisms employed in ~~the~~ recombinant DNA techniques ~~were the bacteria~~, particularly ~~the~~ *Escherichia coli (E.coli)*. In the first ~~tests~~experiments using *E. coli*, strategies similar to those used in the production of synthetic insulin ~~have been~~ were employed. According to these methods, chains A and B were cloned and expressed independently in the host microorganisms ~~in an independent manner~~, thus obtaining two ~~polypeptide~~ polypeptides corresponding to chains A and B. The native insulin was then obtained by ~~carrying out, *in vitro*,~~performing the steps of forming ~~the di-sulphur bridges~~disulfide bonds between chains A and B and the respective intra-~~catenary~~chain bridge *in vitro*,. This oxidizing process ~~were~~

was carried out as ~~it is disclosed~~ described by Chance, R.E. et al., in Diabetes ~~care~~Care 4:147; 1981; and Goedel, D.V. et al. in Proc. Natl. Acad. Sci. U.S.A. 76: 106-110, 1979. One of the ~~most important drawbacks of these methods is~~ biggest difficulties presented by this method was the random formation of ~~the di-sulphur bridges generating~~disulfide bonds, which generated molecules with an incorrect tertiary structure. ~~By~~ Through this method, the yield~~ing~~ of native insulin with biological activity ~~is~~ was extremely low, thus ~~dramatically~~ increasing ~~the~~ production costs~~.~~ dramatically.

~~Considering~~ In view of the above ~~mentioned~~ difficulties~~—~~, experts have introduced the idea ~~has arose in the experts~~ of cloning the DNA sequence ~~corresponding to the~~ of proinsulin or its derivatives ~~wherein~~, where the C peptide ~~C~~ is represented by fragments ~~having several~~ of different sizes. ~~These ides have been~~ This idea is based ~~in~~on the fact that the presence of the C peptide ~~C~~ or its derivatives ~~produced~~ produces a higher yield of ~~proinsulin~~ correctly folded proinsulin after the oxidizing step as compared to the yield resulting from ~~the~~ oxidizing ~~of~~ chains A and B ~~by separate~~ separately (Dteiner, D.F. et al. Proc. Acad. Sci. 60:622; 1968). Thus, it was observed that chain C ~~operating like~~ which acts as a connecting peptide of chains B and A, allows ~~the~~ for cysteine residues ~~are~~ to

be spatially favored for a correct oxidation. It was demonstrated that the ~~thus formed~~ proinsulin molecule formed in this manner could function ~~like~~as a precursor from which insulin could be obtained by ~~removing, *in vitro*, by means of~~ removal of peptide C, using specific enzymes, ~~the peptide C~~. (Kemmler, W. et al. J~~.~~. Biol. Chem. 91: 246:6786; 1971). It was also demonstrated that if ~~the~~ fragment C of these precursors was changed ~~by~~ for a connecting peptide of a smaller size ~~and maintaining~~ that maintains *in vitro* cleavable sites at both ends ~~sites to be split *in vitro* by the~~, and, if proper ~~enzymes~~ enzyme action~~, results~~ was used, equivalent, and in some cases better~~, to the insulin production,~~ results were obtained in the production of insulin. These precursors were named mini-proinsulins (Wollmer, A. et al. Hoppe-Seyler´s, Z. Physiol. Chem. 355:1471-1476; 1974 and ~~EP~~ EPO Patent 195 691).

European Patent No. EP 0055945 discloses a process for producing and expressing proinsulin in *E. coli* and ~~method~~methods for producing human insulin. The production of proinsulin in *E. Coli* on a large ~~scale~~ or commercial scale ~~of proinsulin in *E. coli*~~ is disclosed in US 5,460,954. US 4,431,740 discloses a DNA having a sequence encoding proinsulin, and another DNA encoding pre-

proinsulin, and a microorganism such as *E. coli* transformed with such sequences.

However, the expression of heterologous proteins in *E. coli* has a number of difficulties well known by ~~the experts~~those skilled in the art. Briefly, the following can be mentioned~~.~~:

When an *E. coli* or any ~~other~~ pro-karyotic microorganism is used as a host for the expression of proteins from eukaryotes, the microorganism is incapable of ~~establishing the di-sulphur bridges for permitting~~ forming the disulfide bonds which allow for the correct formation of ~~the~~ a tertiary structure. As a consequence, when proteins such as ~~the~~ human insulin are cloned and expressed in microorganisms, ~~such~~ said proteins tend to aggregate forming inactive complexes or inclusion bodies.

~~The solubilization~~Solubilization and purification of proinsulin from the inclusion bodies requires ~~of~~ a large number of additional steps. One of these steps comprises dissolving the aggregates with reagents such as ~~Urea~~urea or ~~Guanidine~~guanidine chloride. Subsequently, it is necessary ~~subjecting~~to submit the insulin precursor to an oxidizing agent by ~~means of~~ oxidative sulfitolysis, wherein the cysteine molecules of both chains adopt the SSO^-_3 form. Subsequently, the ~~groups~~ S-sulfonated groups are converted into sulphydryl groups (-SH-) in the presence of a

thiolated agent (di-~~thioteitrol~~thiotreitol or 2-mercaptoethanol). Finally, these groups are oxidized in presence of oxygen for ~~forming~~the formation of the sulfide ~~bridges.~~bonds.

~~The~~New methods for ~~recovering~~the recovery of proinsulin from the inclusion bodies are still the aim of several investigations, attempting to improve the yield and ~~the~~ achieve a correct folding of the protein which is dramatically reduced by ~~the~~ purification of the protein and causes the purification process to be extremely complex. (Chance, R. et al. ~~Proceeding~~Proceedings of the Seventh American Peptide Chemistry Symposium, pages 721-728; 1981; Pierce Chemical Company, Rockford, IL.; Chan, S.J. et al. Proc. Natl. Acad. Sci. USA 78 (9): 5401-5405, 1981, and Frank, B.H. et al. ~~Proceeding~~Proceedings of the Seventh American Peptide Chemistry Symposium, pages 729-739; 1981; Pierce Chemical Company, Rockford, IL.).

In addition, ~~with the~~ in *E. coli* or any other prokaryote ~~organism the~~organisms protein translation ~~of the proteins are begun~~starts with a methionine residue. In order to remove the methionine from the amino ~~acid. For eliminating the methionine from the~~ terminal ~~amino~~ end the gene of interest is usually cloned as a fusion protein. The ~~separation~~removal of ~~the~~ insulin from the fusion peptide requires an additional step involving ~~the~~ digestion of the

peptide with specific proteases. Otherwise, the methionine residue must be ~~eliminated by~~ removed with cyanogen bromide (CNBr).

~~The~~ European Patent No. 0 055 945 discloses a method and a vector ~~for splitting~~ to cleave a proinsulin analog~~ous~~ having a smaller C peptide ~~C that is smaller~~ and wherein the methionine residue is ~~eliminated~~removed by ~~employing a~~ treatment with CNBr.

Other difficulties and drawbacks that may be found in the expression of heterologous proteins in prokaryotes is the ~~decreasing~~decrease or ~~diminishing of the~~ reduction of protein stability under the action of ~~the~~ cytoplasmic protease. US Patent No. 5,460,954 discloses a process ~~fro~~ for producing human proinsulin in *E. coli* ~~comprising a~~ which comprises a vector containing a sequence ~~in~~ at the 5′ end of the proinsulin gene ~~of proinsulin~~, encoding an amino acid sequence ~~preventing the~~ which prevents degradation by protease within the cell.

Many investigators are attempting to improve the methods ~~of~~ for producing human insulin in *E. coli* ~~for obtaining~~through a simpler method and with better results. These methods for improving ~~the protein yielding are carried out by~~ protein yield consist in replacing the C peptide ~~C~~ by smaller sequences (Chang, Seung-Gu at al. *Biochem. J.* 329; 631-635, 1998).

Methods for expressing proinsulin in bacteria have also been developed, ~~these methods combining~~ which combine different procedures such as the expression of a fusion protein ~~comprised of a~~ comprising a polyhistidine ~~tale~~tail in the N-terminal end, a methionine residue and the proprotein sequence of ~~the proprotein~~ of ~~the~~ human insulin, all ~~incorporated~~included in an expression vector for bacteria (Cowley, Darrin J. et al. *FEBS Letters*, 402: 124-130, 1997).

~~On the basis~~ By reasonof the operative drawbacks and difficulties found in the expression of human insulin in prokaryotic hosts, many attempts have been made to obtain high ~~expressions~~levels of expression of human insulin in ~~eukaryiotic~~ eukaryotic hosts such as yeast. Consequently, ~~the~~ yeast has become one of the selected hosts for the expression of eukaryotic proteins. These microorganisms provide clear advantages as compared to ~~the~~ bacteria in relation to the production of ~~mammal~~mammalian proteins. ~~The yeast~~Yeast has ~~secretary~~ secretion mechanisms that are similar to ~~the secretary system~~those of ~~the~~ mammals and has the capacity of properly folding, ~~of~~ proteolitically processing, ~~of~~ glycosilating and secreting~~, in a proper manner, the mammal~~mammalian proteins.

When appropriate vectors are employed in the yeast for exporting the protein outside the cell, the process

~~of~~for recovering and ~~purification of~~purifying the proteins exported into the culture medium is simpler and has a better ~~yielding relative to~~yield than the expression in ~~the~~ cell cytoplasm. In addition, the secretion system provides an appropriate environment for the formation of the di-sulfide ~~bridges~~bonds that are necessary for ~~the~~protein folding ~~of the proteins~~ (Smith, et al. 1985; *Science* 229:1219). On the other ~~side, the~~ hand, cytoplasm is a reducing environment ~~wherein these connections~~in which these bonds are not ~~produced~~formed. Under these circumstances, ~~the proteins that need forming~~ production of any proteins requiring di-sulfide ~~bridges~~bonds for maintaining a correct tertiary structure, as ~~it~~ is the case of ~~the~~ insulin, ~~can be produced with~~ will have better results when ~~the same~~ said proteins are secreted.

~~Among the systems employing the~~ yeast ~~for operating~~system used as ~~hosts~~ host for the production of a large number of proteins is, for example, the yeast ~~of the~~ species *Saccharomyces cerevisiae* ~~may be found. The genetics~~. The genetic structure of this yeast has been studied in detail by a number of investigation groups.

Several polypeptides such as ~~the~~ insulin have been cloned and expressed in *Saccharomyces cerevisiae.* The expression of this propeptide may follow the secretory ~~way~~path or may be accumulated in the cytoplasm of the host

microorganism. In the event of the accumulation, time consuming and complex purification processes ~~for purification~~ must be employed, the processes requiring steps for the formation of ~~the~~ di-sulfide ~~bridges~~bonds, as ~~it is~~ disclosed in ~~the~~ European ~~patent~~Patent No. 37255. ~~For avoiding~~In order to avoid these drawbacks and ~~complicate~~complicated steps, the proinsulin gene sequence ~~corresponding to the gene of proinsulin~~ is cloned subsequently to an additional DNA sequence named "leader" or signal peptide that originates the pre-proinsulin peptide. This peptide, once recognized and processed by the yeast, provides the secretion of ~~the~~ proinsulin into the culture medium.

In addition to the foregoing, ~~the precursor~~any precursors of the proinsulin type that are produced in *Saccharomyces cerevisiae* ~~suffer from~~undergo a rapid enzymatic process either when ~~they are~~ expressed in the cytoplasm ~~as well as~~ or when ~~they are~~ secreted into the medium. It has been demonstrated that ~~the~~ human proinsulin is especially sensitive to enzymatic cuts in two dibasic sequences (Arg_{31} - Arg_{32} and Lys_{64} - Arg_{65}). This causes the ~~split~~cleavage of the molecule before the formation of the di-sulfide ~~bridges,~~ bonds, thus resulting in the separate generation of ~~the~~ peptides C, A and B ~~separately~~.

It has been found that if, instead of proinsulin, shorter sequences are employed wherein the C peptide ~~C is~~ has been removed or, it is simply represented by shorter fragments having up to two amino acids of ~~the type of~~ lysine, arginine type, ~~a~~a more stable molecule is obtained ~~that~~, which is ~~more stable,~~ not digestible by proteases, and capable of been processed *in vitro* ~~finally originating~~to give a biologically active insulin molecule (Lars Thim et al. Proc. Natl. Acad. Sci. USA 83: 6766-67770; 1986).

European Patent No. 195 691 discloses several precursors ~~such as of the~~, *inter alia*, those of type B-X-Y-A ~~wherein B and A corresponds to chains~~ where B and A ~~of~~ correspond to the B and A chains of human insulin, and where~~in~~ X and Y are represented by the amino acids lysine and arginine, these amino acids being digestible by the ~~enzymes~~ trypsine and carboxypeptidase B enzymes for ~~its~~ their conversion into human insulin. However, while considerable amounts of A_0Arg-desB(30) are produced as digestion sub-products ~~of the digestion, this~~, these sub-~~product does~~products do not have ~~the~~ amino acid 30 of the B chain ~~and~~while an arginine residue remains connected to the A chain. The arginine residue can not be easily removed ~~thus causing~~and this causes serious inconveniencies in the process of purifying the protein, also considerably

diminishing ~~the production~~ product yields. ~~The total production~~Total yield of this precursor ~~en~~ in *Saccharomyces cerevisiae* is remarkably low.

On the other hand, US Patent No. 4,916,212 discloses a simple~~-~~ chain proinsulin precursor, where~~in~~ said precursor is represented by the formula: $B_{(1-29)}-(X_n-Y)_m-A_{(1-21)}$, ~~wherein~~ X_n is a peptidic chain ~~win~~ of n amino acids, Y is lysine or arginine, n is an integer from 0 to 35, m is 0 or 1, $B_{(1-29)}$ is a B chain lacking the threonine at position 30, and $A_{(1-21)}$ is the A chain of ~~the~~ human insulin. This US Patent ~~discloses~~ reveals that $-X_n-Y-$ ~~as~~does not ~~containing~~ have two adjacent basic amino acids, such as lysine and arginine, because the digestion with trypsin produces ~~sub~~ byproducts that are difficult to separate during the purification steps. The products obtained from these genetic designs do not contain ~~the aminoacid~~ threonine at position 30 and, therefore, they must be subjected to an additional step consisting ~~of~~ in the addition of this amino acid by ~~means of~~ the catalytic action of ~~the~~ trypsin in the presence of ~~the~~ Thr-Obu ester, as ~~it is~~ disclosed in ~~the~~ US Patent No. 4,343,898 and Rose, K. et al. *Biochem. J.* 211:671-676, 1983.

In any case, in addition to all the modifications ~~carried out in the~~ introduced into the insulin precursors, the expression of these peptides in *Saccharomyces*

cerevisiae has resulted in low ~~yieldings~~yields and scaling drawbacks ~~related to the scale of production of~~ in heterologous protein production. These problems are generally associated to low efficiency promoters and to the fact that the sequences of interest are cloned in autonomous replication ~~plasmides. These plasmides are~~plasmids. These plasmids do not ~~kept~~remain uniformly distributed within the culture medium and they usually ~~diminish in~~decrease as the number of copies increases. As a result of this, and after some duplication cycles, cells with 2, 3 or 0 copies of the plasmide used as ~~a~~ vector are found in the culture (Chan, S.J. et al. *Proc. Natl. Acad. Sci.* USA 78 (9): 5401-5405. 1981).

An expression system in yeast, ~~that is distinct from the one~~but not using *Saccharomyces* as a host, is the ~~system of the~~ methylotrophic yeast system. These microorganisms may be very useful as hosts for the expression of heterologous proteins~~, which proteins are~~ required to be produced in large ~~production~~ volumes. ~~The heterologous~~Heterologous proteins that are expressed in methylotrophic yeast may be secreted with expression levels that are equivalent to ~~the ones~~those of *E.coli* and ~~that are~~ higher ~~as compared to the expression levels of the~~ than those of *Saccharomyces cerevisiae*.

~~The methylortrophic~~Mthylotrophic yeasts are unicellular microorganisms capable of growing in the presence of methanol as the only ~~one~~ carbon source. This yeast can be kept without ~~incoveniencies~~trouble in high cellular densities when ~~they are cultured~~grown in a high volume fermentor. In addition, this yeast is capable of producing many of the post-translated modifications ~~carried out by the upper~~undergone by the higher eukaryotic cells, such as proteolytic digestions, protein folding, di-sulfide ~~bridges~~bonds formation and glycosilation.

Pichia pastoris is one of the twelve species within the four yeast ~~genus~~genera capable of metabolizing methanol as the only one carbon source (Cregg, J.M. et al. *Bio/Technology* 11:905-910, 1993). The remaining ~~genus~~genera are represented by *Candida, Hansenula* and *Torulopsis*.

~~This yeast comprise~~These yeasts share a large number of enzymes corresponding to the metabolic pathways of methanol (Veenhuis, M. et al. *Adv. Microb. Physiol.* 24:1-82, 1983). The first step of this metabolic pathway is the ~~oxidizing~~oxidation of methanol into formaldehyde, generating hydrogen peroxide ~~under the~~ by action of the alcohol oxidase enzyme (AOX).

The cell ~~prevents the~~avoids hydrogen peroxide ~~from~~ toxicity by carrying out this first ~~metabolism~~metabolic

reaction of ~~the~~ methanol in a special organelle named peroxisome.

There are two genes in *P. pastoris* ~~encoding~~which encode alcohol oxidase enzymes I and II~~,~~: AOX1 and AOX2 genes. The AOX2 gene is responsible of ~~the~~ most ~~part of the~~ alcohol oxidase activity in the cell (Cregg, J.M. et al. *Mol. Cell. Biol.* 9:1316-1323, 1989).

The expression of this gene is highly regulated and it is induced by ~~the~~ methanol, with ~~the~~ AOX1 representing a value close to ~~the~~ 30% of the total soluble ~~proteins of the~~ cell~~. Because of this~~ proteins. It is for this reason that the expression systems ~~most~~usually employed ~~in~~ with *Pichia pastoris* include in their vectors the AOX1 gene promoter.

Thomas Kjeldsen et al. ~~carried out a comparison between~~ compared the expression of proinsulin and precursor peptides of (B_{1-29} - Ala-Ala-Lys - A_{1-21}) insulin in *S. cereviciae* and in *P. pastoris*. The products were secreted ~~in~~into the culture medium ~~through~~ with the aid of an amino acid sequence that is fused to the leader (amino ~~termination~~) end of the precursor~~ termed leader. For~~. Several signal peptides were employed for determining the secretion efficiency of the insulin precursor ~~several signal peptides were employed~~ such as the pre-pro-peptide α mating factor of *Saccharomyces cerevisiae* and synthetic derivatives thereof. These pre pro peptides have amino acid

sequences useful ~~for target for the action from~~ as targets for the activity of specific proteases ~~permitting the liberation~~ resulting in the release of the peptide into the culture medium. All the insulin peptides employed by these authors are secreted into the medium as a precursor lacking threonine at position 30 of the B chain. This product, recovered and purified from the culture medium, had to be ~~subject~~ submitted to an exhaustive process called transpeptidation. ~~The transpeptidation~~Transpeptidation consists ~~of~~ in the addition of threonine and it is disclosed in US Patent No. 4,916,212 to Markussen et al. ~~The transpeptidation includes an additional~~It adds a step ~~in~~ to the purification process of the insulin molecule.

In the above described state of the art, it has been a concern of the inventors to find a solution to all of the above mentioned problems and drawbacks in the prior art.

SUMMARY OF THE INVENTION

It is therefore one object of the present invention to provide a new yeast strain capable of producing and secreting into the medium an insulin precursor in proper quantities useful for ~~its~~ industrial application, wherein the inventive strain comprises two distinct DNA

constructions for expressing a DNA sequence encoding an insulin precursor. Said gene is cloned in such a way that an insulin precursor is secreted into the medium, ~~the~~ said insulin precursor containing at its ~~termination~~terminal end ~~the~~ a first amino acid ~~corresponding to~~ of the insulin B-chain, thus avoiding the steps ~~for eliminating~~of removing the remaining ~~amino~~amino acids from the signal peptide.

It is a further object of the present invention to provide a methylotrophic recombinant yeast strain for producing human insulin precursor, the strain having a genome comprising a copy of a first DNA construction and a second DNA construction, wherein said constructions control~~ling~~ the expression and secretion of a human insulin precursor, said DNA constructions comprising at least one DNA sequence encoding a human insulin precursor or analogues thereof.

It is even another object of the present invention to provide a yeast strain comprising in its genome DNA constructions capable of expressing a human insulin precursor of the formula:

B(1-30)-Y1-Y2-A(1-21), wherein Y1 is lysine or arginine; Y2 is lysine or arginine; B(1-30) is the B peptide of ~~the~~ human insulin; and B(1-21) is the A peptide of human insulin.

It is even another object of the present invention to provide a *Pichia pastoris* strain deposited on July 25, 2000, ~~in the~~with American Type Culture Collection (ATCC) under accession number PTA-2260, wherein the yeast strain comprises, in its ~~genome~~genome, a first DNA construction comprising:

a) a first ~~insertable~~ insertion DNA sequence corresponding to a 5′ regulatory region of *Pichia pastoris* AOX1 gene, operably linked to

b) the MF α signal sequence of *Sacharomyces cerevisiae*, ~~operable~~operably linked to

c) the sequence encoding a human insulin precursor, preferably a precursor of formula B(1-30)-Y1-Y2-A(1-21) ~~operable~~operably linked to

d) a 3′ transcription termination sequence of *Pichia pastoris* AOX1 gene operably linked to

e) a *Pichia pastoris* HIS4 ~~selection~~selectable gene operably linked to

f) a second insertion sequence corresponding to the 3′ end of the *Pichia pastoris* AOX1 gene ~~3′ end~~; and

a second DNA construction comprising:

a) a first ~~insertable~~insertion DNA sequence corresponding to a 5′ regulatory region of *Pichia pastoris* AOX1 gene operably linked to

b) the MF α signal sequence of *Sacharomyces cerevisiae* ~~operable~~operably linked to

c) the sequence encoding a human insulin precursor, preferably a precursor of formula B(1-30)-Y1-Y2-A(1-21) ~~operable~~operably linked to

d) a 3′ transcription termination sequence of the *Pichia pastoris* AOX1 gene operably linked to

e) the zeocine-resistant ~~selection~~selectable gene.

It is still another object of the present invention to provide a first DNA construction comprising at least one expression cassette for expressing the human insulin precursor, the cassette comprising:

a) a 5′ regulatory region operably linked to

b) a DNA sequence encoding a signal sequence ~~operable~~operably linked to

c) a sequence encoding a human insulin precursor ~~operable~~operably linked to

d) a functional 3′ transcription termination sequence.

According to an embodiment of the invention, the first DNA construction comprises, at its 5′ and 3′ ends, sequences ~~homologous~~ with sufficient homology to a target gene of the yeast ~~enough~~ to permit the insertion by gene replacement of the DNA construction in the target gene, in the same relative ~~orientation~~sense of the target gene in

the yeast genome, these 5′ and 3′ sequences that are homologous to the target gene being sequences flanking the expression cassette.

It is even another object of the present invention to provide a

first DNA construction ~~of claim 5~~, further comprising:

a) a first ~~insertable~~ insertion DNA sequence corresponding to a 5′ regulatory region of *Pichia pastoris* AOX1 gene operably linked to

b) the MF α signal sequence of *Sacharomyces cerevisiae* ~~operable~~operably linked to

c) the sequence encoding a human insulin precursor, preferably a precursor of formula B(1-30)-Y1-Y2-A(1-21) ~~operable~~operably linked to

d) a 3′ transcription termination sequence of *Pichia pastoris* AOX1 gene operably linked to

e) a *Pichia pastoris* HIS4 ~~selection~~selectable gene operably linked to

f) a second insertion sequence corresponding to the 3′ ~~termination~~ sequence of the *Pichia pastoris* AOX1 gene.

It is a further object of the present invention to provide a second DNA construction comprising at least one expression cassette for expressing ~~the~~ a human insulin precursor, the cassette comprising:

a) a 5′ regulatory region operably linked to

b) a DNA sequence encoding a signal sequence ~~operable~~operably linked to

c) a sequence encoding a human insulin precursor ~~operable~~operably linked to

d) a functional termination sequence.

According to a preferred embodiment of the invention, the second DNA construction comprises a selection marker gene distinct from the selection marker gene of the first DNA construction, thus permitting a second selection of the inventive transformed yeast strain.

According to another embodiment of the invention, the second DNA construction comprises a single sequence homologous enough with a target gene of ~~the~~ yeast, allowing the integration of the DNA construction ~~in~~into the target gene, in a single event.

It is even another object of the present invention to provide a second DNA construction comprising:

a) a first ~~insertable~~insertion DNA sequence corresponding to a 5′ regulatory region of *Pichia pastoris* AOX1 gene operably linked to

b) the MF α signal sequence of *Sacharomyces cerevisiae* ~~operable~~operably linked to

c) the sequence encoding a human insulin precursor, preferably a precursor of formula B(1-30)-Y1-Y2-A(1-21) linked to

d) a 3´ transcription termination sequence of *Pichia pastoris* AOX1 gene linked to

e) ~~the~~a zeocine-resistant ~~selection~~selectable gene.

According to an embodiment of the invention, in both DNA constructions, the sequence encoding the human insulin precursor is cloned in said construction ~~following the site of~~ in a position adjacent to the protease site, wherein all the secreted human insulin precursor contains, in its amino terminal region, the ~~fenilalanine~~phenylalanine amino acid.

Also according to an embodiment of the invention, each of the DNA constructions is incorporated into a vector selected from the group consisting of linear and circular vectors.

It is even another object of the present invention to provide a method of obtaining a transformed methylotrophic yeast strain for producing high quantities of a human insulin precursor, the method comprising the steps of:

i) transforming a yeast cell with a first DNA construction comprising:

a) a first ~~insertable~~ insertion DNA sequence corresponding to a 5′ regulatory region of *Pichia pastoris* AOX1 gene operably linked to

b) the MF α signal sequence of *Sacharomyces cerevisiae* ~~operable~~operably linked to

c) the sequence encoding a human insulin precursor, preferably a precursor of formula B(1-30)-Y1-Y2-A(1-21) ~~operable~~operably linked to

d) a 3′ transcription termination sequence of the *Pichia pastoris* AOX1 gene operably linked to

e) a *Pichia pastoris* HIS4 ~~selection~~selectable gene operably linked to

f) a second insertion sequence corresponding to the 3′ end of the *Pichia pastoris* AOX1 gene;

ii) selecting the yeast cells;

iii) isolating a yeast strain;

iv) re-transforming the yeast strain obtained in steps i)-iii) with a second DNA construction comprising:

a) a first ~~insertable~~insertion DNA sequence corresponding to a 5′ regulatory region of the *Pichia pastoris* AOX1 gene operably linked to

b) the MF α signal sequence of *Sacharomyces cerevisiae* ~~operable~~operably linked to

c) a sequence encoding a human insulin precursor, preferably a precursor of formula B(1-30)-Y1-Y2-A(1-21) linked to

d) a 3′ transcription termination sequence of the *Pichia pastoris* AOX1 gene linked to

e) the zeocine-resistant ~~selection~~selectable gene;

v) selecting the re-transformed yeast strain; and

vi) isolating the selected and re-transformed yeast strain.

It is still another object of the present invention to provide an insulin precursor secreted into the medium as a precursor containing threonine at position 30 of B chain, thus avoiding the complex and cumbersome transpeptidation step.

The above and other objects, features and advantages of this invention will be better understood when ~~taken~~interpreted in connection with the accompanying drawings and description~~.~~ thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention is illustrated by way of example ~~in~~with the following drawings where~~in~~:

FIG. 1 ~~provides~~shows a restriction map of plasmid pPIC9-Ib; and

FIG. 2 ~~provides~~shows a restriction map of plasmid pPICZαA-Ib;

DESCRIPTION OF THE PREFERRED EMBODIMENTS

Unless otherwise defined ~~in another way~~ herein, the technical and scientific terminology ~~utilized~~used in the present description has the meaning ~~as~~ commonly ~~interpreted~~understood by the person skilled in the art ~~of the invention~~. All ~~the~~ patents and publications mentioned in the present application are incorporated herein ~~as a~~by reference only.

Definitions

The term "human insulin precursor" or "proinsulin" as used herein refers to any human insulin precursor or analog~~ue~~ thereof originating an insulin molecule, or related molecules showing the same biological activity ~~of the~~ as insulin.

The meaning of "biological activity" is the biological activity associated ~~to~~ with insulin ~~evaluated through~~, as measured by tests known ~~for the person~~ by those skilled in the art.

As used herein, ~~the~~ "insulin precursors" include the allelic variations of ~~the~~ insulin precursors and derivatives obtained through simple modifications of the amino acid sequence of ~~the~~ an insulin product.

As used herein, the terms "leader sequence" or "signal sequence" are ~~indistinct~~equivalent expressions and refer to amino acid sequences ~~carrying out~~ involved in the transport of a peptide linked thereto through the cellular membrane.

As used herein, the term "~~a~~ DNA construction" ~~encircle~~ refers to an expression cassette and ~~also~~ other DNA sequences.

In order to ~~re~~solve the above mentioned drawbacks and problems, the inventors have developed a new and inventive yeast strain expressing high quantities of a human proinsulin molecule. This yeast strain ~~has been~~ was obtained by a new and inventive process or method comprising the steps of sequentially transforming and re-transforming the yeast with two ~~distinct~~ different and inventive DNA constructions. The proinsulin secreted into the medium by the new strain is an insulin precursor~~, said precursor being preferably a precursor~~ with its C peptide ~~C~~ ~~being~~ replaced by a sequence of two amino acids ~~and wherein the~~, whose purification ~~of same, for obtaining~~ into the active human insulin~~,~~ generates few contaminants, thus avoiding the ~~Trypsin~~trypsin-mediated transpeptidation steps without ~~diminishing~~reducing the required industrial yields or production. Also, said DNA constructions ~~have been~~ was cloned in such a way as to avoid the step of

~~eliminating~~removing the remaining amino acids of the signal peptide ~~of~~from the secreted proinsulin.

~~By using~~Using the strain and ~~the~~ DNA construction of the present invention it is possible to obtain production levels of human insulin ~~between~~ from 200 to 400 mg/liter of fermentation ~~are obtained~~broth, such levels being considered very appropriate for industrial production.

The chain encoding the human insulin precursor gene has been obtained through synthesis, ~~by employing~~using the polymerase chain reaction.

The gene synthesis ~~methodology~~ process has the advantage of ~~being rapid~~taking a short time and ~~permitting~~also of allowing for the selection of the ~~codons~~ most ~~utilized~~ frequently used codons by the selected expression host.

This process comprises the chemical synthesis of ~~an oligonucleotide~~ a group ~~composing~~of oligonucleotides that forms the entire sequence of both DNA chains of the selected precursors. Subsequently, ~~the linking of~~ complementary ~~oligonucleotide pairs is carried out. For preventing~~oligonucleotides are paired. In order to prevent the problems associated ~~to cross~~ with cross'hybridization events between the oligonucleotides, a PCR method ~~has been~~

~~employed, this method permitting to complete the process~~was used, and the process was completed in only one day.

The first step consists ~~of the production of a~~ in providing a central template. ~~Oligonucleotides located~~The oligonucleotides were placed in the center of the sequence to be ~~construed~~constructed, the oligonucleotides being complementary to each other in their 3´ ends, ~~having an~~ with a specific *Tm* for each pair of nucleotides.

Subsequently, after elongation by PCR, from the 3' End, a complete double-stranded chain ~~has been~~was obtained ~~by PCR from the 3´ end~~.

An aliquot of the PCR mixture ~~has been~~was employed in a second PCR event after the addition of the corresponding primers.

Subsequently, the process was continued with appropriate pairs of primers until ~~obtaining~~ the final product was obtained.

Once the DNA fragments encoding the human insulin precursor ~~have~~had been obtained, the fragments were ~~incorporated~~inserted into vector pPIC9 (~~Invitro). Once both entities were linked~~*In vitro*). After ligation, the recombinant vector was characterized ~~with~~by restriction ~~enzymes~~enzyme assays. Some of the randomly taken recombinant vectors were sequenced according to the Sanger method ~~employing~~using the ~~Kit~~ Sequenace V 2.0. Kit The

primers ~~employed~~used for ~~the~~ sequencing ~~of~~ the 5′-3′ strand ~~5′-3′~~ were 5′ AOX1 and factor α, ~~and for~~while the ~~strand 3′-5′ the primer~~ 3′ AOX1 primer was ~~employed~~used for the 3′-5′ strand, both ~~primer~~ primers being ~~provided~~supplied by Invitrogen. The results of ~~the sequence~~ sequencing confirmed that, the proinsulin sequence in the vector~~, the sequence of the proinsulin~~ was correct.

The ~~new~~ newly formed vector was termed pPIC9-Ib (Fig.1). The vector digested with the appropriate restriction enzyme ~~originated~~formed two DNA fragments~~; the~~. The fragment containing the inventive DNA construction was ~~employed~~ used for transforming the methylotrophic yeast. Said DNA construction comprises a methanol responsive sequence represented by the AOX1 gene promoter element of a methylotrophic yeast, a DNA sequence encoding a signal sequence, a human insulin precursor gene, the transcription termination signal sequence of the AOX1 gene, and the HIS4 gene encoding histidinol dehydrogenase, all ~~contained~~ of them included between 5′ and 3′ ends of the AOX1 gene.

According to the invention, as it is well known ~~for~~ by any person skilled in the art, any circular or linear integrative site-specific vector may be ~~utilized~~used for the transformation of the yeasts.

In the DNA construction according to the invention, ~~also~~ any signal sequence permitting the proper exportation of the insulin precursor may also be ~~utilized~~used. Preferably, the ~~signal sequence~~ MF α signal sequence of *S. cerevisiae* ~~that~~, which is a peptide ~~comprised~~made up of 13 amino acid residues - may be employed. The MF α signal sequence has a protease site determined by the sequence of amino acids Lys-Arg-Glu-Ala (~~SEQ~~DEQ ID NO:26). During the cloning process in pPIC9 of a human insulin precursor gene, this gene ~~may be~~is preferably inserted into the ~~site~~ Xho I ~~that eliminates~~ site removing the -Glu-Ala- residues, whereby the starting insert of the human insulin gene is maintained immediately ~~after~~ adjacent to the proteases removal site (Figure 1). The cloning in ~~site~~ Xho I ~~permits to obtain~~site allows for the obtention of a precursor which is released into the culture medium without the remaining amino acids ~~belonging to~~of the signal peptide, thus simplifying the steps of purification of ~~the~~ human insulin.

There are several genes ~~in the yeast which are~~ included in the methanol metabolism pathway in yeast. The expression of these genes is controlled by their regulatory 5′ regions which are responsive to methanol and are known as promoters. Any of ~~such~~ said regulatory 5′ regions are ~~proper for utilization~~ appropriate for use as promoters in the DNA construction according to the invention. Examples

of regulatory regions include, but are not limited ~~thereto~~, the *Pichia pastoris* primary alcohol oxidase enzyme (AOX1) gene promoter, the secondary alcohol oxidase II enzyme (AOX2) gene promoter, the *P. pastoris* dihydroxyacetone synthase (DAS) gene promoter, the *P. pastoris* ~~p40~~P40 gene promoter, the *P. pastoris* catalase gene promoter, and the ~~glyceraldehide~~glyceraldehyde P dehydrogenase GAP promoter. Preferably, the *Pichia pastoris* primary alcohol oxidase enzyme (AOX1) gene promoter may be ~~employed because this is~~ used since it is highly efficient ~~to provide~~in providing high levels of expression. It will be apparent ~~for~~ to any person skilled in the art that any of the promoters or regulatory regions selected are within the scope of the invention, the 5′ regions being preferred, however, ~~the preferred ones because~~by reason of their capacity ~~of being responsive~~to respond to an alcohol-containing medium.

The 3′ termination sequences of the DNA construction according to the invention are ~~proper~~ appropriate for terminating, ~~polyadenylation~~polyadenylating and stabilizing the ~~RNAm~~mRNA encoded by the insulin precursor gene. ~~termination~~Termination sequences that are characteristic of methylotrophic yeast families ~~may be employed~~, preferably termination sequences of *Pichia pastoris* 3′~~ termination sequences~~′, may be used.

The DNA construction also contains a selectable marker gene. For these purposes, any ~~selection~~selectable marker gene ~~may~~can be ~~employed~~used provided that the gene is functional in methylotrophic yeast including, but not limited ~~there~~to, any gene capable of providing a selected phenotype to the methylotrophic yeast, ~~permitting positively selecting the yeast~~ which allows for the positive selection of the yeasts transformed with the DNA construction of the invention. An appropriate marker is any system ~~utilizing a~~ comprising a mutant auxotrophic *Pichia pastoris* host cell and the wild biosynthetic type gene complementing the defficiencies of the host ~~defects. Preferably,~~. It is preferable to use the HIS4 gene encoding ~~the~~ histidinol dehydrogenases and ~~the~~ auxotrophic mutant ~~cell may be employed.~~cells.

~~The~~ A particular feature of the DNA construction of the invention ~~employed~~used for transforming methylotrophic yeasts ~~has the characteristic of being insertable~~is that it can be inserted into the genome of the host yeast through homologous recombination with the 5′ and 3′ ends of the endogenous AOX1 gene of ~~the~~ yeast, ~~wherein~~ said endogenous gene ~~is~~ being replaced by the DNA construction of the invention.

The DNA construction according to the invention ~~may~~can be inserted ~~in~~ into any functional vector in

bacteria (chimeric vector), wherein the vectors include selection markers and replication sites ~~proper~~appropriate for the bacteria. ~~This vector may have a~~ These vectors can be of circular shape forming extra-chromosomal replication plasmids within the bacteria. Several copies of the inventive DNA construction ~~could~~may be incorporated into said vector.

The ~~inventive DNA construction is employed~~DNA constructions of the invention are used for transforming methylotrophic yeasts according to any standard method of ~~transforming yeasts~~yeast transformation. Examples of ~~transforming~~ transformation methods include, ~~while they~~ but are not limited to ~~the following~~, electroporation, spheroplasts, transformation with lithium chloride and transformation with PEG 1000; preferably, the ~~method of spheroplasts~~spheroplast and electroporation ~~are employed. The transformation~~methods. Transformation with the DNA construction can be carried out with the DNA construction arranged in linear ~~o~~ or circular pattern. The DNA construction is directed to the target gene of the yeast genome by flanking sequences ~~having~~ with enough homology with the target gene ~~in order~~ so that the DNA construction ~~is~~can be integrated into the site to which it ~~was~~ is directed. In ~~a different~~ an additional embodiment of the invention at least one copy of the DNA construction

according to the invention is integrated into the host genome in the correct orientation.

It is ~~also~~ possible to employ any ~~other~~ methylotrophic yeast strain. Examples of methylotrophic strains include, ~~while~~ but are not limited ~~there~~to, the ~~genes~~ genera *Pichia, Torulopsis, Hansenula* and *Candida*~~, with~~. It is preferable to use the *Pichia pastoris* GS 115 strain (ATCC N~~°~~O 20864) ~~being preferably employed,~~, as this strain ~~having~~ contains the mutated HIS4 gene, and therefore, it is HIS^-.

~~With~~ Of all ~~the~~ His^+ ~~transformers the inventive~~transformants, integration of the DNA construction ~~integrated by replacing~~of the invention by replacement of the structural AOX1 gene of the GS115 strain genome occurs with a frequency of about 5% to 35%. The replacement event of the structural AOX1 gene of the yeast genome generates yeasts called Mut^s, which are sensitive to the use of methanol as a carbon source. Any expert in the art can understand that the ~~inventive~~ DNA construction of the invention can also be integrated ~~by some~~through one of its 5′ or 3′ ends within the AOX1 gene generating Mut^r yeasts which are resistant to the use of methanol as a carbon source because they keep the functional AOX1 gene; the DNA construction could also be integrated ~~with the genome of~~into the yeast genome by recombination with the

His yeast gene ~~of the yeasts the~~whose sequence ~~of which~~ is also present in the DNA construction of the invention, or the DNA construction ~~could~~can be integrated ~~in~~into several sites of the yeast genome without restricting the scope of the invention.

Subsequently, the clones transformed with the ~~inventive DNA construction~~DNA construction of the invention are selected by any method known in the art but ~~preferably replica~~it is preferable to perfom replicate plating experiments ~~are carried out in permitting~~which make it possible to distinguish between His^+ Mut^r clones and His^+ Mut^s clones. Alternatively, clones with the capability of production can be selected ~~by employing~~using electrophoretic ~~geles~~gels and immunochemical techniques.

Each ~~of~~ Mut^s ~~/~~ and Mut^r ~~clones~~ clone selected by the above mentioned methods ~~was~~ were sub-cloned and isolated as pure clones. ~~Among~~From all the selected clones, those producing ~~proper~~adequate quantities of the insulin precursor were selected. These selected clones were characterized and the number of copies of the ~~inventive~~ DNA construction of the invention was ~~analyzed.~~determined. Several clones producing ~~proper~~adequate quantities of the insulin precursor were detected, ~~with~~and some of them ~~being~~ were Mut^s ~~and other ones being~~ while others were Mut^r.

~~Some~~Two of these clones were ~~employed for subjecting the same to the~~ subjected to a second transformation event herein also called ~~also as~~ re-transformation.

In another embodiment of the invention, the nucleotide sequence ~~of nucleotides~~ encoding an insulin precursor was amplified by PCR, isolated and cloned in the pPICZαA vector in the especially designed multi-cloned site ~~and~~, thus obtaining a vector called pPICZαA-Ib (Figure 2) ~~was obtained~~. Said vector contains a new DNA construction, called second DNA construction, comprising a promoter responsive to methanol of the methylotrophic yeast AOX1 gene; a DNA sequence encoding a signal sequence, the insulin precursor gene, ~~the~~ a transcription termination signal sequence ~~of the termination of the transcription~~ and a selection gene ~~distinct from~~other than the ones ~~employed~~used in the first DNA construction of the invention.

Any signal sequence ~~appropriately permitting the sequencing of~~ which allows for the appropriate secretion of the insulin precursor may be employed. Examples of signal sequences include, ~~although they~~but are not limited to ~~the following~~, the MFα signal sequences of *s. Cerevisiae* and the signal sequence of alkaline phosphatase~~, with~~. It is preferable to use the MFα signal sequence of *s. cerevisiae*,

corresponding to a peptide having 13 amino acid residues~~, being preferably employed~~. During the cloning process of the gene encoding the insulin precursor in the pPICZαA plasmid, said gene was inserted ~~in~~into the Xho I site ~~that~~which eliminates (the -Glu-Ala- residues, whereby the ~~beginning of the~~ insulin precursor gene ~~remained~~was placed immediately ~~after~~adjacent to the protease~~s~~ removal site. ~~This~~ With this cloning design ~~permits~~ the precursor released into the culture medium ~~to be~~ is free of ~~remaining~~residual amino acids of the signal peptide, thus avoiding one step in the purification sequence of ~~the~~ human insulin.

Any 5´ regulatory sequence ~~is proper for employing~~can be used as a promoter in the second DNA construction of the invention. Examples of regulatory regions include, ~~while they~~but are not limited ~~there~~to, the *Pichia pastoris* primary alcohol oxidase enzyme (AOX1) gene promoter, the secondary alcohol oxidase II enzyme (AOX2) gene promoter, the *P. pastoris* dihydroxyacetone synthase (DAS) gene promoter, the *P. pastoris* p40 gene promoter, the *P. pastoris* catalase gene promoter, and the ~~glyceraldehide~~glyceraldehyde P dehydrogenase GAP promoter. ~~Preferably,~~ The preferred regulatory region is the *Pichia pastoris* primary alcohol oxidase enzyme (AOX1) gene promoter ~~may be employed because this is highly efficient~~

~~to provide~~ by reason of the high levels of expression obtained therewith. It will be apparent ~~for~~to any person skilled in the art that any of the selected promoters or regulatory regions ~~selected~~ are within the scope of the invention. ~~Preferably, the~~The preferred 5′ regulatory regions ~~that~~ are capable of ~~being responsive to an alcohol~~ responding to a methanol-containing medium ~~are employed~~.

The 3′ termination sequences of the second DNA construction according to the invention are ~~proper for terminating~~appropriate for the termination, polyadenylation and ~~stabilizing the RNAm~~stabilization of the mRNA encoded by the insulin precursor gene. ~~termination~~Termination sequences that are characteristic of methylotrophic yeast families may be employed~~,~~; preferably~~—~~, *Pichia pastoris* 3′ termination sequences are ~~employed~~used.

The second DNA construction of the invention also contains a selectable marker gene. For these purposes any ~~selection~~selectable marker gene functional in methylotrophic yeasts may be ~~employed~~used, but it must be different from ~~the one employed~~that used in the ~~prior~~above transformation. ~~It is~~ The preferred ~~to employ~~ marker gene is the zeocine gene ~~encoding~~which encodes for resistance to the zeocine antibiotic~~s~~.

The new vector for ~~the~~ re-transformation of the yeasts may comprise a single copy or multiple copies of the

second DNA construction of the invention. Any method for obtaining a vector with multiple copies may be ~~employed, but preferably a method comprising~~used, but the preferred method comprises a strategy ~~of~~ for cloning ~~multimerics is employed, generating~~multimers, which generates a vector with multiple copies of the second DNA construction of the invention, preferably containing ~~between~~from 2 ~~and~~ to 18 copies of said DNA construction.

The new isolated recombinant vectors ~~are~~ were characterized by ~~means of~~ analysis with restriction enzymes. The recombinant vectors were sequenced and the correct position within the vector of the sequence encoding the insulin precursor and the signal peptide ~~has been~~ were confirmed~~; and~~, as well as the number of copies of the gene of interest ~~has also been determined.~~.

The new vectors ~~have been employed~~were used for ~~the~~ re-transformation of the yeasts. Vectors containing ~~between~~from 1 ~~and~~ to 18 copies of the insulin precursor gene may be ~~employed. Preferably, a~~used. A preferred vector ~~containing~~contains a single copy of the second DNA construction of the invention ~~may be utilized~~. Said recombinant vector may be linearized by digestion with a restriction enzyme or may be ~~utilized~~used in the circular form for re-transforming the yeasts cells. Preferably, the ~~linearized vector is utilized for the carrying out of the~~

second transformation event in the Mut^s clones obtained in the prior transformation step~~.~~ is carried out by said linearized vector.

~~Any~~ Re-transformation may be carried out by method known in the art for the transformation of yeasts~~ may be utilized in the re-transformation, these methods including, although they~~. These methods include, but are not restricted to~~ the following~~, spheroplasts, ~~electropoarione~~electroporation, transformation with PEG 1000 and transformation with lithium chloride. ~~Preferably, the~~The preferred methods are spheroplasts ~~transformation method or the~~and electroporation~~ are utilized.~~.

Any ~~expert~~person skilled in the art can understand that, ~~for the carrying out of the second transformation event, vector~~vectors with ~~a~~ single or multiple copies of the gene of interest may be employed~~,~~ in the second transformation event and wherein said retransformation step can be carried out by employing any method of ~~transforming yeasts~~ yeast transformation without restricting or modifying the scope of the present invention.

The ~~vector~~ linearized vector with the ~~inventive~~ DNA construction ~~utilized~~of the invention used for re-transforming the methylotrophic yeasts clones ~~producing~~which produce the insulin precursor may preferably ~~have the feature of~~ be capable of being inserted into the

host genome in only one site, subsequently generating multiple genomic copies *in vivo*.

The re-transformed clones ~~have been properly~~ were appropriately selected by employing any of the known methods ~~in order to carry out~~ of double selection of yeasts~~, preferably, employing a~~. Preferably, double selection is carried out in a medium without histidine and with zeocine.

The selected positive clones were isolated and purified, and the sequences integrated into the yeast~~s~~ genome were characterized. The presence, in the total DNA, of re-transformed yeast~~s~~ clones of the ~~inventive~~ DNA constructions of the invention were determined ~~employing~~ by the Southern blot method and by ~~means of a~~ genomic analysis by PCR. The number of copies of the DNA construction of the present invention in the yeast~~s~~ genome ~~has been~~ was determined by ~~employing~~ the well-known Dot Blot method and ~~by means of the method of~~ analyzing the number of copies by PCR.

~~Among~~The selected clone from all the characterized clones was the B1,~~—~~3.3~~.3~~ clone~~—~~. This clone was deposited on July 25, 2000 ~~in~~ with the American Type Culture Collection (ATCC) under deposit number PTA-2260, ~~was preferably selected, the clone containing~~and contains a copy of the first DNA construction of the invention and 13

copies of the second DNA construction of the invention, wherein said clone is Mut^s, it is resistant to ~~the~~ zeocine and it can grow in a histidine--free medium.

Other ~~production clones with the following features were~~ isolated production clones were: C1,--46 clone: of phenotype Mut^r, wherein the integration of the first DNA construction of the invention was in the His yeast gene, containing 5 copies of the DNA construction, and wherein said clone was ~~subject to~~ transformed in a single transformation event; C2,--7 clone: of phenotype Mut^s, containing a single copy of the DNA construction and where~~in~~ said clone was ~~subject to~~ transformed in a single transformation event; clone 25; of phenotype Mut^r, containing 6 copies of the DNA construction, the integration of the construction was in the yeast AOX 1 gene, and where~~in~~ said clone was ~~subject to~~ transformed in a single transformation event; clone V8,-10.1: of phenotype Mut^s, with 8 copies of the second DNA construction of the invention generated *in vitro,* and where~~in~~ said clone appears after ~~a~~ the re-transformation event of a clone containing at least one copy of the first DNA construction.

All the transformed and retransformed strains selected ~~by~~ for their desired phenotypic and genotypic ~~desired characteristic were cultured~~ characteristics were grown in Erlenmeyer flasks. The colonies and ~~strain~~

~~resulting~~ strains of interest were selected to be ~~cultured~~grown in fermenting devices.

~~For a large~~Large scale production of ~~the~~ insulin precursors~~,~~ was performed using the typical ~~method~~methods and processes used for methylotrophic yeasts ~~were utilized~~; preferably, ~~the~~ fermentations were carried out ~~by culturing~~growing the yeast~~s~~ strains in the first step in a medium ~~having~~ containing an excess of a non inducing carbon source ~~in excess, like~~, such as glycerol. In this step, ~~the~~ expression of the ~~inventive constructions~~constructions of the invention with the gene encoding the human insulin precursor is totally ~~repressed, generating~~ supressed, with generation of an important biomass but without ~~insulin precursor expression~~producing the gene of interest. Subsequently to this ~~growing~~ period of growth the cells were grown preferably under methanol ~~restricting~~restrictive conditions with or without another carbon source for inducing the expression of the desired gene contained in the DNA constructions of the invention. Said DNA constructions were capable of expressing the gene encoding a human insulin precursor ~~in reply to the~~as a response to methanol and were also capable of releasing or secreting significant ~~quantities~~amounts of ~~the~~ precursor into the ~~culturing~~culture medium, ~~in quantities enough and appropriate to be employed in the~~being said amounts

appropriate and sufficient to be used on an industrial scale.

The present invention will now be described with reference to certain examples which further illustrate but do not limit the invention.

EXAMPLES

Example 1

Construction of the insulin precursor

~~The construction of an~~An insulin precursor ~~has been carried out~~was constructed by ~~means of~~ the Polymerase chain reaction (PCR), employing human codons:

~~The~~ PCR conditions ~~have been~~were established ~~according to the details of the publication~~as described in: A. Method for Synthesizing Genes and cDNAs by Polymerase Chain Reaction. Di Donato, Alberto et al. Analytical Biochemistry. 212:291-293; 1993, modifying the annealing temperature according to the Tm of each oligonucleotide.

Primers:

SEQ ID~~: N°~~ NO:1: 5´-TCACACCTGG TGGAAGCTCT CTACCTAGTG

TGCGGG -3´

SEQ ID~~:~~ ~~N°~~ NO:2: 5′-GGTCTTGGGT GTGTAGAAGA AGCCTCGTTC

_CCCGCACACT AGGTA-3′

SEQ ID~~:~~ ~~N°~~ NO:3: 5′- TTTGTGAACC AACACCTGTG CGGCTCACAC CTGGTGGAA -3′

SEQ ID~~:~~ ~~N°~~ NO:4: 5′-GCTGGTACAG CATTGTTCCA CAATGCCACG

CTTGGTCTTG GGTGT -3′

SEQ ID~~:~~ ~~N°~~ NO:5: 5′-CTAGTTGCAG TAGTTCTCCA GCTGGTAGAG

GGAGCAGATG CTGGTACAGC AT-3′

Final Product:

SEQ ID~~:~~ ~~N°~~ NO:6: 5′- TTTGTGAACC AACACCTGTG CGGCTCACAC CTGGTGGAAG CTCTCTACCT AGTGTGCGGG GAACGAGGCT TCTTCTACAC ACCCAAGACC AAGCGTGGCA TTGTGGAACA ATGCTGTACC AGCATCTGCT CCCTCTACCA GCTGGAGAAC TACTGCAACT AG -3′

(complete insulin precursor)

Example 2

Construction of an insulin precursor by ~~the~~ polymerase chain reaction (PCR) ~~with~~using the codons ~~more utilized by~~most commonly found in Pichia pastoris.

Primers:

SEQ ID~~: N°~~ NO:7: 5´-ACTTGGTTGA AGCTTTGTAC TTGGTTTGTG GTGAAAGAGG TTTCTTCTAC-3´

SEQ ID N~~°~~O:8: 5´-AGAAGTACAA CATTGTTCAA CGATACCTCT CTTAGTCTTT GGAGTGTAGA -3´

SEQ ID~~:N°~~ NO:9: 5´-ACACTTGTGT GGTTCTCACT TGGTTGAAGC TTT-3´

SEQ ID~~:N°~~ NO:10: 5´- TTACTCGAGT TAGTTACAGT AGTTTTCCAA TTGGTACAAA GAACAGATAG AAGTACAACA TTGTTC -3´

SEQ ID~~: N°~~ NO:11: 5´-CCGCTCGAGA AGAGATTTGT TAACCAACAC TTGTGT -3´

The ~~obtained~~resulting product contains the following sequence:

SEQ ID~~: N°~~ NO:12:

5´-TTTGTTAACC AACACTTGTG TGGTTCTCAC TTGGTTGAAG CTTTGTACTT GGTTTGTGGT GAAAGAGGTT TCTTCTACAC TCCAAAGACT AAGAGAGGTA TCGTTGAACA ATGTTGTACT TCTATCTGTT CTTTGTACCA ATTGGAAAAC TACTGTAACT AA-3´

The PCR conditions were identical to ~~the ones of~~ those in Example 1.

1- ~~The~~One twentieth part of the product ~~obtained in~~ from each PCR was employed as a template for the subsequent event.

2- The final PCR product was purified ~~by~~in a microspin S300 column (Amersham) and digested with the Xho I restriction enzyme.

The digestion product was ligated to the pPIC9 vector ~~that was~~ previously digested with the restriction enzyme Xho I.

3- A digestion with the Hpa I restriction enzyme was carried out ~~for detecting~~in order to detect the recombinant clones and the correct orientation of the insert.

Example 3

Construction of Factor α ~~with preferences~~ using the codons ~~of~~most commonly found in Pichia pastoris

~~By means of this technique the~~ The nucleotide sequence corresponding to the leader sequence or signal peptide was cloned~~.~~

~~The employed primers were~~ using the ~~following~~same method.

The primers were:

SEQ ID~~: N°.~~ NO:13: 5´-CGCGGATCCA AACCATGAGA TTCCCATCTA TCTTCACTGC TGTTTTGTTC GCTGCT -3´

SEQ ID~~: N°.~~ NO:14: 5′- GTTTTGTTCG CTGCTTCTTC TGCTTTGGCT GCTCCTGTTA ACACTACTAC TGAAGACGAA ACTGCTCA-3′

SEQ ID~~: N°.~~ NO:15: 5′-ACGTCGAAGT CACCTTCCAA GTCAGAGTAA CCGATAACCG CTTCAGCTGG GATTTGAGCA GTTTCGTCTT C - 3′

SEQ ID~~: N°.~~ NO:16: 5′-GATGAACAAC AAACCATTAT TAGTAGAGTT AGAGAAAGGC AAAACAGCAA CGTCGAAGTC ACCTTC -3′

SEQ ID~~: N°.~~ NO:17: 5′-CCGCTCGAGA GAAACACCCT CTTCCTTAGC AGCGATAGAA GCGATAGTAG TGTTGATGAA CAACAAACCA TT - 3′

The final product has the following sequence:

SEQ ID N°O:18

5′-ATGAGATTCC CATCTATCTT CACTGCTGTT TTGTTCGCTG CTTCTTCTGC TTTGGCTGCT CCTGTTAACA CTACTACTGA AGACGAAACT GCTCAAATCC CAGCTGAAGC GGTTATCGGT TACTCTGACT TGGAAGGTGA CTTCGACGTT GCTGTTTTGC CTTTCTCTAA CTCTACTAAT AATGGTTTGT TGTTCATCAA CACTACTATC GCTTCTATCG CTGCTAAGGA AGAGGGTGTT TCTCTCGAGA AGAGAGAGGC TGAAGCA-3′

Cloning of the MFα and the insulin precursor with ~~preferences~~the most commonly used codons of *Pichia pastoris*:

1- The signal peptide pPIC9 was replaced by a signal peptide with *Pichia pastoris* codons. pPIC9 was digested with restriction enzymes BamHI y XhoI

2- The digested fragments were separated ~~in~~on 0,8% agarose gel and the 7780 bp~~.~~ fragment was recovered.

3- The PCR product SEQ ID~~: N°~~ NO:18 was digested with the same restriction enzymes ~~utilized~~used in 1 and was ligated to the fragment obtained in 2.

4- The vector obtained in 3 and the PCR fragment SEQ ID N~~º~~O:12 ~~was~~were digested with ~~the~~ XhoI, and subsequently they were ligated.

5- The recombinants ~~having~~with the correct orientation of the insulin precursor insert were detected ~~by~~using the HpaI.

EXAMPLE 4

Cloning of an insulin precursor gene in a pPIC9 yeast~~s~~ vector

The DNA fragment encoding the insulin precursor was amplified by PCR, employing as a template SEQ ~~N°~~ID NO:6

obtained previously ~~obtained~~, and as primers the following sequences:

SEQ ID~~: N°~~ NO:19. 5´ -GGGATCCAT ATGCTCGAGA AAAGATTTGT GAACCAACAC CTGT-3´.

SEQ ID~~: N°~~ NO:20. 5´ -TTAGAATTCC CGGGTCTAGT TGCAGTAGTT CT- 3´.

The ~~obtained~~resulting PCR product was purified ~~by employing the~~using a DNA ~~clean up system~~Clean Up System Kit (Promega), according to the ~~manufacturer~~manufacturer's instructions.

The JM-109 *E. coli* strain was transformed with vector pPIC9.

Subsequently, the plasmid DNA was removed by using the Wizard ~~plus miniprep~~Plus Miniprep DNA ~~purification system~~Purification System Kit (Promega).

The vector and the insert were digested with Xho I and Eco RI and both molecules were ligated according to conventional protocols.

5 µl of the ligation product were ~~utilized~~used for transforming 100 µl of competent bacteria ~~corresponding to~~of Jm-109 *E. coli*. strain according to conventional protocols.

The DNA was recovered from the ~~colonies~~ ampiciline-resistant ~~to ampiciline by~~colonies using the above disclosed method.

200 ng of DNA of each sample were digested with 5 U of Alw NI restriction enzyme or with 5 U of Xho I and Eco RI enzymes.

The colonies containing the recombinant plasmids were grown and the plasmid DNA was recovered and purified.

Subsequently, the plasmid DNA was sequenced. The primers employed in the sequencing of ~~strand~~ the 5′ - 3′ strand were ~~the following~~: 5′ AOX1 and α-Factor. The ~~strand~~ 3′ - 5′ strand was sequenced by means of ~~the~~ primer 3′ AOX1 (sequences provided by the ~~Kit from~~ Invitrogen~~, called~~ Kit, and designated 3′AOX1, 5′AOX1 and α-Factor.

The DNA required for this sequence was purified ~~by means of~~with a miniprep SV Kit (Promega). ~~Between~~ 3 - 5 µg of DNA were employed ~~per~~for each sequencing and the ~~employed~~ protocol was that ~~one~~ suggested by the Amersham Kit.

Example 5:

Cloning strategy for an insulin precursor in a pPICZαA yeasts vector.

~~In this~~ This example~~,~~ illustrates the cloning of a copy of a gene encoding ~~the~~a human insulin precursor~~,~~ in pPICZαA~~, is illustrated~~.

The selected vector is ~~the~~ pPICZαA, the general map thereof being shown in figure 2. This vector has 2 XhoI

sites ~~XhoI~~, one ~~of them being~~ located in the multiple cloning site (1185) ~~while~~and the other ~~one is located~~ in position 1247. The vector was digested with XhoI and the gene of interest was cloned according to the following protocol:

PPICZαA	10 µl (≅ 2µg)
~~Buffer~~ Neb2 (10x) Buffer	4 µl
H_2O	23,6 µl
BSA (100 X)	0,4 µl
Xho I	2 µl (40 U)

~~The digestion~~Digestion was carried out at 37° C for 6 hours. 40 µl of digestion product were ~~applied~~ loaded into a ~~column of~~ HR S-200 microspin column (Amersham).

Subsequently, ~~a dephosphatizing~~dephosphatation was carried out with intestinal alkaline phosphatase or CIP according to the following protocol:

pPICZαA (digested)	40 µl
~~buffer~~ NebCIP (10x) buffer	5 µl
H_2O	4 µl
CIP	1 µl

The reaction was carried out at 37° C for 30 minutes. Finally, the reaction was stopped by heat (75°C

for 10 minutes) and the DNA was purified by applying the ~~same~~ sample into a ~~column of~~ microspin HR S-400 column.

Insert preparation

The insulin precursor was amplified by PCR ~~by utilizing~~using the same conditions ~~employed~~as in example 4 corresponding to the cloning of this sequence in vector pPIC9 with the following primers:

SEQ ID~~: N°~~ NO:19: 5′ - GGGGATCCAT ATGCTCGAGA AAAGATTTGT GAACCAACAC CTGT-3′

SEQ ID~~: N°~~ NO:21: 5′-TCACTCGAGC GGTCTAGTTG CAGTAGTTCT-3′

50 µl of PCR product were purified ~~by applying to~~in a ~~column of~~ microspin HR S-200~~.~~ column. The product was digested for 6 hours according to the following protocol:

PCR products	40µl (≅600 ng)
~~Buffer~~ Neb2 (10 X) Buffer	5 µl
H_2O	3 µl
BSA (100 X)	0,5 µl
Xho I	1,5 µl (40 U)

~~The digestion~~Digestion was stopped by heat (65° C, for 20 minutes) and the digestion products were purified ~~by~~ in a ~~column of~~ microspin HR S-200~~.~~ column.

~~The ligation~~Ligation of the insulin precursor fragment to the ~~to the vector~~ pPICZαA vector was carried out according ~~with~~to the following protocol:

The vector and the insert were digested with Xho I and were again quantified ~~for carrying out the~~ prior to ligation. ~~The ligation~~Ligation was carried out with 100 ng of vector in each event, ~~by utilizing~~using the following molar relations of vector/insert 1:1, 1:3, 1:6 and 1:0 (negative control).

~~Bacteria *E. coli* of~~ Top 10 strain of *E. coli* bacteria (Invitrogen), ~~were~~ was transformed with 5 µl of each of the above mentioned ~~relations.~~ratios. ~~In~~Thirteen colonies were obtained on the plate corresponding to ~~relation~~ratio 1:3, ~~13 colonies were obtained vs~~while 6 colonies were obtained in the negative control. ~~13~~Said thirteen colonies were picked in tubes with 1,5 ml of LB medium for preparing conventional minipreps. The ~~obtained~~resulting DNAs were digested with ~~de~~restriction enzyme AlwNI ~~for determining~~to determine the number and orientation of the ~~obtained~~resulting recombinants.

~~7~~

Seven recombinant colonies were obtained, ~~with two of them~~2 in the correct orientation, thus ~~obtaining the~~resulting in vector pPICZαA Ib (Figure 2).

The DNA of one of the colonies was ~~utilized~~used for transforming the TOP10 strain.

Example 6:

Multimeric cloning strategy of a human insulin precursor in a yeast vector.

This example ~~discloses the~~describes a method for obtaining ~~of~~ a multicassette containing multiple copies of the gene encoding ~~the~~an insulin precursor in the vector pPICZαA Ib obtained in Example 5.

The process ~~followed~~used to obtain the construction with two copies of the gene of interest is ~~that one disclosed,~~described below as ~~a multimerics generating protocol~~ an *in vitro* multimeric generation protocol according to the detailed instructions provided by the manufacturer (Invitrogen)~~, as follows~~:

Two digestions were carried out:

Digestion 1: pPICZαA Ib with Bam HI

Digestion 2: pPICZαA Ib with Bam HI and Bgl II.

The expression cassette was recovered from an agarose gel.

The ~~cassette~~ Bgl II-BamHI cassette , containing a copy of the insulin precursor gene was ligated with the product from digestion 1, and the ~~bacteria *E. Coli*~~ Top 10 ~~were~~ strain of *E. Coli* bacteria was transformed.

The plasmid DNA was ~~removed~~extracted and the presence of recombinants was ~~analysed~~analyzed by restriction mapping.

The two types of configurations were differentiated by restriction mapping with Bgl II and Bam HI. ~~Those configurations in~~ The direct tandem configurations were chosen ~~for continuing~~to continue the process. ~~By means of this process a~~ A vector ~~called~~designated pPICZαA Ib2 was generated~~, the vectors having the ends~~ using this process, with compatible Bgl II and Bam HI ~~compatibles~~ends for ~~the~~ ligation. However, both sites ~~are~~ were destroyed when ligated.

~~The protocol of~~The in vitro multimeric generation ~~of multimerics *in vitro*~~protocol was ~~again~~ employed ~~by~~again, but replacing ~~the~~ vector pPICZαA Ib ~~by the~~ for vector pPICZαA Ib2, thus obtaining ~~the~~ vector pPICZαA Ib4 (vector with 4 copies of the gene in direct tandem).

Finally, a vector pPICZαA Ib8 was generated ~~from~~with the prior protocol by replacing ~~the~~ vector pPICZαA ~~Ib4 by the~~Ib for vector pPICZαA Ib4.

~~For~~To obtain ~~of~~ the ~~cassette~~ BglII-BamHI cassette, 4 μ~~l~~g of ~~the~~ DNA were digested with both enzymes simultaneously, at 37°C, overnight. ~~Then~~Subsequently, a gel was run with 0~~,~~.8% agarose (Promega), ~~in~~ such ~~a way~~ that the cassette was separated with the ~~multimerics~~multimers of

the remaining vector. ~~For purifying~~To purify the DNA fragment ~~of~~from the agarose, the corresponding band of the gel was ~~split~~cut out and it was purified according to the ~~protocol of~~Promega Clean-up Kit ~~from Promega.~~protocol.

The recombinant clones were always ~~were~~ detected with the ALwNI enzyme.

Example 7:

Yeast~~s~~ transformations:

The ~~chosen~~ strain selected for ~~the~~ transformation was *Pichia pastoris* GS115 *(His4)* (Invitrogen).

The transformation process was carried out according to the protocols of the instruction manual (~~pichia~~Pichia expression Kit; version G161219, 250043) provided by Invitrogen.

Spheroplasts transformation process:

100 ul of spheroplast preparation (disclosed by Invitrogen) were ~~utilized~~used for each transformation event, and 10 ug of DNA (pPIC9-Ib) were added to the preparation. This preparation was incubated for 10 minutes at room temperature. During ~~the~~ incubation, 1 ml of 1:1 PEG/CaT solution was added to the cell~~s~~ and DNA solution. This preparation was ~~homogenised~~homogenized and incubated for 10 minutes at room temperature.

After a centrifugation step at 750 X g for 10 minutes, the cellular pellet was re-suspended in 150 ul SOS medium and was kept for 20 minutes at room temperature. Then, 850 ul of 1M sorbitol were added and the cells were plated ~~in~~on agarose.

Several volumes (100 - 300 ul) of spheroplasts transformed with 10 ml of RD molten agarose were mixed and poured over plates containing RDB medium. Each sample was ~~carried out~~made by duplicate.

The plates were incubated at 28 - 30°C for 4 - 6 days. Samples were taken and ~~the~~ cellular viability was determined by ~~cultivating~~growing the yeast~~s~~ cells in a RDHB medium containing histidine.

Example 8

Selection and isolation of recombinant yeasts

The ~~transformations of~~ transformation of *Pichia pastoris* GS115 yeast~~s~~ strains with vector pPic9 digested with *Bgl* II promotes the recombination in ~~the~~ locus AOX I. ~~The replacement~~Replacement of the structural alcohol oxidase (AOX 1) gene occurs with a frequency of 5-35% of the ~~transformers~~ His^+~~.~~ $^+$ transformants.

~~By means of~~ A plate replication experiment ~~in plates~~ on ~~a~~ minimum medium containing dextrose (MD) and ~~a~~ minimum medium containing methanol (MM), ~~transformers~~was

used to distinguish transformants Mut$^+$ and Mut^s ~~can be distinguished.~~.

~~Colonies~~ His$^+$ colonies from the transformation of example 7 were selected according to the following protocol:

Each colony was picked with a sterile tip and ~~was~~ applied ~~on~~to a MM plate by ~~making a mark~~marking or ~~strake~~streaking, and then ~~over~~ to a MD plate.

In order to differentiate both phenotypes the corresponding controls ~~to~~ for GS115/His$^+$ Mut$^+$ and GS115/His$^+$ Mut^s (Invitrogen) were included.

The plates were incubated at 30° C for 48 - 72 hours. ~~This method permitted to distinguish~~ By this method it is possible to identify the clones Mut^s as well as those Mut^r that normally ~~grows in plate~~ grow on plates MD and MM.

Each of the clones Mut^s and Mut^r selected ~~by~~using this method was purified and pure clones were isolated. ~~The isolation~~Isolation was carried out by ~~effecting strakes~~making streaks of each colony ~~in~~on a minimum medium ~~without~~lacking histidine.

Example 9

Re-transformation of yeast~~s~~ clones obtained in Example 8

~~The re~~Re-transformation of clones was carried out ~~by employing~~using the electroporation transformation method according to the protocol suggested by Invitrogen. The DNA ~~utilized~~used in the transformation corresponds to 20 µg of plasmid pPICZαA Ib.

Example 10

Identification and isolation of colonies producing insulin precursor, retransformed as in example 9.

~~Once the~~After retransformation ~~was finalized~~, the presence of clones producing the insulin precursor was revealed by means of an immunochemical method.

Aliquots of 50 to 600 ul of transformed cells were spread ~~in~~on plates containing YPDS agar medium with 100ug/ml Zeocine.

Once the colonies resistant to Zeocine were grown, the presence of the insulin precursor was detected according to the following scheme:

On each plate under ~~analyses~~analysis a nitro cellulose membrane was placed in such a way that the membrane was in contact with each of the colonies and it was also ~~deposited in~~applied as an inverted form ~~unto~~onto the ~~culturing~~culture plates containing BMMY/agarose medium.

The plates were incubated with the filters ~~adhered~~attached for 24 hours at 30_°C.

Then, the membranes were removed and subjected to ~~washing~~ a series of washes with a solution of ~~PBS/~~0.05% to 0.1% PBS/Tween-20 for an hour, changing the medium regularly.

The nitrocellulose membranes were blocked with 5% skimmed milk in ~~PBS/~~0.1% PBS/Tween-20 for 1 hour at 4°C.

Subsequently, the membranes were incubated with a ~~policlonal~~ Guinea pig anti~~body anti~~-human insulin~~,~~ polyclonal antibody for one hour at room temperature, and then ~~were~~ washed with ~~PBS/~~0.1% PBS/Tween-20 solution for 30 minutes.

Subsequently, the filters were incubated with ~~an anti~~ Guinea pig anti-IgG polyclonal antibody conjugated with peroxidase for 1 hour at room temperature and the filters were washed with a ~~PBS/~~0.1% PBS/Tween-20 solution for 30 minutes. Finally, the presence of peroxidase was revealed with 0.012% H_2O_2 , 0.08% DAB; 100mM Tris/~~ClH~~ClH, pH 7.5.

The resulting positive colonies ~~that resulted positive~~ were identified and isolated from the original plate.

Based on the comparison of the reaction intensities, the high ~~producing~~yielding clones were selected.

Example 11:

Expression of the recombinant clones

In order to ~~determining~~determine the productivity of the selected colonies, growing and induction experiments with BMGY/BMMY ~~medium~~media were carried out. The first ~~culturing~~culture medium contains glycerol ~~that is~~ as the carbon source ~~utilized for~~ used by the microorganism for producing biomass. The second culture medium contains methanol ~~that~~which is the inductor of AOXI promoter.

The colonies were grown in Erlenmeyer flasks in a BMGY medium at 30°C until ~~reaching~~ an OD_{600nm}: 6 - 20~~.~~ was reached. Then, the cells were centrifuged ~~for replacing~~in order to replace the cultured medium ~~by~~ using BMMY in a volume corresponding to ~~the~~ a fifth ~~part~~ of the volume ~~utilized~~used in the growing phase. ~~The culturing~~Culturing was continued for 120 hours ~~as from~~ after the medium change at a temperature of 30°C with stirring. ~~Each~~Every 24 hours 0.5% v/v 100% methanol was added and samples were taken to be evaluated by electrophoresis ~~in~~on 15% ~~polyacrilamide~~polyacrylamide, Tris/Tricine gel. Each sample was centrifuged ~~for removing~~to remove the cells, and the supernatant was treated with a sample buffer according to the protocols provided by (Laemmli, U.K. *Nature* 227:680-685; 1970).

~~From the polyacrilamide gels those clones~~ Clones capable of secreting a peptide with an electrophoretic ~~movility coincident~~motility consistent with that ~~one for~~of the insulin precursor having a PM ~~of between~~ from 5~~.~~,800 to 5~~.~~,900, were ~~chosen.~~selected from the polyacrylamide gels.

The ~~chosen~~selected clones ~~shown~~showed a very high protein expression. ~~Subsequently, the~~Thus, molecular characterization of the ~~producing clone~~ genome ~~was carried out~~of said clones was performed.

Example 12

Molecular characterization of recombinant clones

The extraction of *Pichia pastoris* DNA was carried out according to the method suggested by the Invitrogen guide.

Southern Blot Analysis

The Southern Blot analysis was carried out according to the standard protocol.

Briefly, ~~a 871 bp fragment~~an AOX probe ~~that~~ which is a fragment of 871 bp of the AOX1 promoter obtained from the digestion of vector pPICZαA with enzymes Bg1II and HindI was ~~utilized; and it was also utilized the following:~~

used. The His probe ~~that is~~, a fragment of 1587 ~~bp~~pb of the HIS4 gene obtained by digestion of vector Ppic9

with the MscI; and the Ins probe ~~that~~which is a fragment of 227 ~~bp~~pb obtained by PCR employing as a template the plasmid PPIC9IB and the primers corresponding to the sequences SEQ ID: ~~15~~19 and ~~16.~~20, were also used.

The chromosomal DNA was digested with the BglII~~.~~ enzyme.

In ~~the~~ filters hybridized with the AOX probe ~~the~~ a band of about 1600 bp corresponding to AOX1 endogen gene was observed, not only ~~in~~ for the non transformed GS115 yeasts but also ~~in the~~ for other Mut^r clones. However, this band ~~did~~ was not ~~appear in the~~ observed for Mut^s transformed clones. In all transforming clones a band of 5700 bp corresponding to the expression cassette of insulin precursor under promoter AOX1 and the HIS4 gene was observed~~, coming~~. This resulted from ~~the~~ transformation with ~~the~~ BglII-digested pPIC9-Ib ~~digested~~, showing varying intensities depending on the number of inserted copies. In some clones, other bands with ~~BglII, with distinct intensity depending from the number of incorporated copies. In some clones, other bands having distinct~~varying sizes were observed and these bands could correspond, for example, to ~~the~~ Bg1II sites lost ~~of sites Bg1II by exonucleases previous to the~~ through exonucleolytic cleavage before insertion ~~in~~into the chromosome. In ~~the~~ clones ~~coming~~resulting from the retransformation of clone

C2,7 with PPICZαA-Ib (clone B1, 3.3) (linearized with SacI), in addition to the band of 5.7 kbp, a band of 3.8 kbp corresponding to the insertion of the insulin precursor cassette under control of AOX1 promoter, and the zeocine gene was also observed.

Detailed analysis of each clone hybridized with the AOX probe.

Pichia pastoris GS115: expected band of 1.6 kbp

Clone 25: expected ~~band~~ bands of 1.6 and 5.7 kbp

Clone C1,46: expected ~~band~~ bands of 1.6 and 5.7 kbp plus 3 bands of 7.8; 7.3 and 4.8 kbp.

Clone C2,_7: expected band of 5.7 kbp. ~~Absent~~Lacks band of 1.6 kbp, indicating that this is a Mut^s ~~transformer.~~transformant.

Clone B1,_3.3: band of 5.7 kbp (insertion of C2,7) plus band of 3.8 kbp corresponding to insertion of pPICZαAIb cassette (linearized with SacI).

The same filters ~~utilized~~used with the AOX probe were re-hybridized with the HIS probe. A band of 2.7 kbp corresponding to ~~HIS4~~ endogenous HIS4 gene was observed, not only in the non transformed yeasts GS115 but also in most of the transforming clones. This band ~~did~~ was not ~~appear~~observed in clone C1,-46 indicating in this case that there was an integration at the level of this gene ~~thus~~

~~loosing the~~ whereby the BglII sites ~~BglII.~~were lost. In all the transforming clones a band of 5700bp was observed corresponding to the insulin precursor cassette under the AOX1 promoter and a HIS4 gene ~~coming~~resulting from the transformation with BglII-digested pPIC9-Ib ~~digested with BglII, with different intensity~~, showing varying intensities depending on the number of incorporated copies. In some clones, other bands ~~having different~~of varying sizes were observed, ~~this~~ for example bands corresponding ~~for example~~ to the ~~lost~~loss of ~~sizes~~ BglII ~~by~~sites through exonuclease ~~digests previous to the~~ digestions prior to insertion ~~in~~into the chromosome.

By the analysis of the Southern Blots hybridized with the HIS probe, a clone with only one copy of the expression cassette was individualized which ~~clone was~~was then taken as a pattern for the further determination of the number of copies in ~~the~~ other clones.

By ~~the~~ hybridization of the membranes with the insulin probe it was confirmed that all the bands obtained by hybridization with AOX and HIS ~~probe excepting~~probes except— those corresponding to the endogenous genes, contained the gene corresponding to the insulin precursor.

Dot Blot Analysis

~~For determining~~In order to determine the number of copies ~~of~~ in the sequence of the insulin precursor of the ~~several~~different transforming clones, the Dot Blot technique was employed, using Ins and Gap probes. The Gap probe was ~~employed~~used as a single copy gene pattern in all the clones. The number of copies of insulin was determined on the basis of~~From~~ the relation~~ship~~ between the signals obtained with ~~both probes~~each probe and ~~taking as a reference an~~ using the insulin single copy clone as a reference (obtained by ~~analysis of~~ Southern Blot Analysis), ~~the number of copies of insulin was determined~~.

The number of copies of the gene encoding the insulin precursor in each clone was the following:

Clone 25: 6

Clone C1,_46: 6

Clone C2,_7: 1

Clone B1,_3.3: 13

Clone V8,_10.1: 8

Characterization of Mut^s or Mut^r colonies by PCR:

A protocol according to the following scheme was ~~utilized~~used:

Chromosomal DNA:	10-20mg
5´AOX	0,_5 µM
3´AOX IN	0,_5 µM

dNTP 0,_2 mM

C12Mg 1,_5 mM

Taq: 2U

Buffer 10X ~~1~~1 x

Sequence of primers:

5´AOX I: 5´ - GACTGGTTCC AATTGACAAG C (~~provided by Invitrogen)~~

SEQ ID~~: N°~~ NO:25)

(3´ AOX IN): 5´ - GTCGTGGTTT CTCATAGTAG AGTGGACA (SEQ ID NO:22)

The reaction conditions were the following: Denaturalization 94°C 2 minutes. 1 cycle

25 cycles:

Denaturalization 94°C, 1 minute.

Annealing 55, 1 minute.

Extension 72°C, 1 minute.

Final extension: 72°C, 7 minutes. 1 cycle

~~The~~A band of 730 bp ~~appears~~was observed in Mut^r clones. No band is observed in Mut^s clones.

Quantification of the number of copies of the insulin precursor gene by PCR in the recombinant colonies.

DNA was extracted from all the samples and the quantity was normalized by means of PCR with Gap primers

(~~gliceraldehyde,~~glyceraldehyde 3-~~phosfate dehidrogenase~~phosphate dehydrogenase, single copy gene).

A new PCR was carried out with ~~primers~~ insulin specific ~~for insulin~~ primers according to the prior quantification, ~~by utilizing the analyzing~~using increasing concentrations of templates for each point. In this way, ~~the signal~~ saturation of the signal was avoided.

The PCR product was analyzed ~~in~~on a ~~gel of~~ 2% agarose gel and after staining with ~~a etidio~~ethidium bromide the bands were visualized with ~~an~~a Fotodyne imaging equipment ~~for taking images fotodine.~~. Quantification ~~has been~~ was carried out with ImageQuant software.

~~As a unit the clone~~Clone C2,7 was ~~chosen, the unit having~~ selected as a unit, which has a single copy of the insulin precursor gene while the remaining clones were compared according ~~with~~ to the intensity of the PCR products.

~~For guarantying~~In order to make sure that the experimental conditions of the ~~amplification~~amplifications of the Gap genes and insulin ~~was~~were equivalent, primers ~~have been designed having~~with a similar hybridization T° and similar sizes were designed.

Gap primers:

~~SEQ ID: N° 23 (Gap5´): 5´ GGTCATCACT GCTCCATC~~

~~SEQ ID: N° 24 (Gap3´): 5´ AGCAGCACCA GTGGAAGAT~~

Gap5: 5´ GGT CAT CAC TGC TCC AT (SEQ ID NO:23)

Gap3´: 5´ AGC AGC ACC AGT GGA AGA (SEQ ID NO:24)

PCR conditions:

Denaturalization: 94°C, 3 minutes

24 cycles of:

94°C, 1 minute

56°C, 1 minute

72°C, 30 seconds

Chromosomal DNA: 0,_5 - 1,5 NG

Gap primers 5´: 0,_5 µM

Gap 3´: 0,_5 µM

dNTP: 0,_2 mM

ClMg: 1,_5 mM

Taq Pol: 2,_5 U

Buffer 10x: 1 x

The conditions for the insulin precursor were the same ~~to the above~~ as described ~~with~~above for the specific primers.

Insulin precursor primers:

SEQ ID~~: N°~~ NO:19: 5´- GGGGATCCAT ATGCTCGAGA AAAGATTTGT GAACCAACAC CTGT

SEQ ID~~: N°~~ NO:21: 5´- TCACTCGAGC GGTCTAGTTG CAGTAGTTCT

The results from the ~~experiments by~~ Dot Blot and quantitative PCR experiments were ~~equivalent~~consistent, in

other words, the same number of copies of ~~obtained~~ recombinants was found ~~for~~in both methodologies.

Example 13: Fermentation process

F~~The f~~ermentation was carried out not only in a ~~fermentor~~ BioFlo 3000 (New ~~Brinswick~~Brunswick Scientific) but also in a Biostat II (B.Braun Biotech~~).~~) fermentor. Both fermentors are provided with 2,5 ~~liters glasses.~~liter vessels. However, the fermentation process may be adapted to ~~higher~~greater volumes ~~protocols~~.

Culture preparation

The pre-culture for inoculating the fermentor was carried out in 125 ml Erlenmeyer ~~125 ml~~ flasks with 25 ml of BMGY culture medium, ~~the same~~which was inoculated ~~with~~ in the corresponding strain~~,~~ from frozen samples in 50% glycerol at - 80°C.

The culture was incubated at 30°C, 240 r.p.m. for 14 hours in an orbital ~~stirrer~~shaker.

Fermentation

The ~~total~~entire volume of 25 ml was transferred to the fermentor containing 1.2 L of BSM basal medium plus 4.35 ml/l of trace salts and 1% glycerol. The temperature was ~~controlled~~set to 30°C, ~~the~~ dissolved oxygen ~~was~~

~~dissolved~~ at 35%, ~~the~~ pH ~~was~~ of 4.5 and ~~the~~ aeration was of 1 vvm. ~~The dissolved~~Dissolved oxygen was controlled ~~by means of the variation~~ according to PID control of the stirring ~~velocity~~speed and addition of O_2. The pH was controlled by ~~means of~~ automatic addition of a 28% ammonium hydroxide solution.

After approximately 16 hours of ~~culturing,~~ culture, or when the optic density reached ~~the~~a value close to 20 ~~unites~~units of ~~absorbency~~absorbance at 600 nm, the ~~lots culturing~~ batch culture phase was ~~finalized.~~completed.

The ~~lot~~batch phase ~~was~~ begun by feeding the fermentor ~~by means of the addition of~~with an additional 50% glycerol plus 12 ml/l of trace salts. ~~The velocity of addition~~ The addition rate was regulated at 24 ml/l/h. This phase lasted approximately 20 hours, reaching values of OD~~:~~ of 300.

Once the growing phase of the biomass was ~~finalized~~completed, the cells ~~remained~~were kept without feeding for half an hour and the production phase ~~was~~ begun. During said phase ~~the~~ pH was regulated ~~between~~ from 3.5 ~~and~~ to 5.5, and 100% methanol was ~~feed~~ added plus 12 ml/l of trace salts, at ~~the velocity~~a rate of 1.2 ml/l/h. This last phase can be extended for up to 96 hours. Variations ~~in the choosing of the~~may be introduced by selecting adequate ~~time~~times for adding methanol to the

culture, ~~variations in the~~ changing methanol concentration and ~~variations in the inclusion of the~~using a double ~~feeding~~ feed of glycerol/methanol, ~~may be carried out~~ for further improving the production process.

~~Once~~After this step ~~was finalized,~~, the process is complete and the step of ~~separation~~separating the cells from the culture ~~was~~broth is begun. When the fermentation process was carried out with high volumes, appropriate separation methods were ~~utilized.~~used.

The implementation of this fermentation protocol in 1 and 100 liters ~~permitted to obtain between~~ allowed for the obtention of 200 ~~and~~ to 400 mg of insulin precursor per liter of fermentation according to the ~~quantity~~amount of methanol employed ~~in~~for the induction.

The supernatant was ~~apt for being introduced in~~appropriate for the first step of the purification of the insulin precursor.

Example 14:

Purification of recombinant Human Insulin

Capturing the precursor

~~The recovering~~Recovery of the recombinant human insulin precursor from the culture medium was carried out by ~~means of~~ cation ~~interchange~~exchange chromatography, for example, SP Sepharose Fast Flow (Pharmacia) (Katsoyannis,

P. G., ~~y col.~~et al. *Biochemistry* 6:2642-2655; 1967) or by means of another adsorptive chromatographic technique, such ~~us~~as for example ~~the~~ Hydrophobic Interaction Chromatography ~~by utilizing~~using a Phenyl Sepharose Fast Flow resin, according to the protocols disclosed by Gagnon, Pete et Al. Large Scale Process Development for Hydrophobic Interaction Chromatography, Part 1: gel Selection and Development of Binding Conditions. BioPharm 8:21-29; 1995.

The washing Buffer consisted of a solution of 50 mM sodium acetate and 50 mM NaCl, and the elution buffer consisted of 50 mM sodium acetate and 450 mM NaCl. The precursor was maintained soluble by the addition of ethanol or urea.

During the process, the column was ~~equilibrated~~ balanced by 3 Vc of washing buffer ~~as~~at a lineal ~~velocity~~rate of 100 cm/h. B~~The linked~~inding of the product was carried out at a linear~~l~~ ~~velocity~~rate of 90 to 120 cm/h. Once this step was ~~concluded, a washing~~completed, the product was rinsed with 4 Vc of washing buffer ~~was carried out.~~. The product was eluted with 10 Vc of eluting buffer. The chromatographic process was monitored by OD at 280 nm. Those fractions containing the product of interest were collected in a single solution.

Enzymatic processing of the insulin precursor

Digestion with trypsine and carboxipeptidase B

The digestion was carried out by adjusting the concentration of the precursor solution ~~between~~ from 1 ~~and~~ to 20 mg/ml, ~~according to what is~~ as disclosed in the European Patent N~~°~~O EP 195691. The reaction ~~prosecution~~progress was monitored by ~~means of~~ RP-HPLC. D~~The d~~igestion was ~~stop~~stopped with 7.5 M acetic acid.

Proteases were ~~eliminated~~removed from the reaction medium by molecular exclusion (chromatography or ionic ~~interchange~~exchange chromatography at pH: 2-5). The fractions corresponding to the digested precursor were collected in a single solution for its further digestion with carboxipeptidase B according the European Patent EP 195691.

As an alternative method, the simultaneous addition of both enzymes was carried out ~~by~~ following the protocols disclosed in the European Patent EP 195691 and in the publication Lila R. Castellanos-Serra et Al. *FEBS Letters* 378: 171-176; 1996.

F~~The f~~inal purification of the insulin ~~obtained after~~resulting from the enzymatic action can be carried out by any chromatographic technique such as the ones disclosed in ~~the~~ US Patent N~~°~~O 5~~.~~,663~~.~~,291; EPO N~~°~~O 195691; and the techniques disclosed in the publication of Kroeff; Eugene et Al. Journal of Chromatography. 461:45-61: 1989.

While preferred embodiments of the present invention have been illustrated and described, it will be ~~obvious~~evident to those skilled in the art that various changes and modifications may be made therein without departing from the scope of the invention as defined in the appended claims.